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(54) Title: NUCLEIC ACID SEQUENCES ATTACHED TO MATERIALS SENSITIVE TO MAGNETIC FIELDS, AND METHODS OF ASSAY AND APPARATUS USING SUCH ATTACHED SEQUENCES.

(57) Abstract

Magnetic particles coated with cellulose derivatives, e.g. nitrocellulose, link as a support to DNA or RNA sequences. A probe sequence, itself labelled, e.g. with biotin, hybridizes with suitable complementary supported sequences, and the resulting complexes are magnetically separated. Avidin, or streptavidin, then couples with the biotin and the complex is again magnetically separated. The avidin can be already linked with a marker enzyme such as horseradish peroxide, or glucose oxidase, or alkaline phosphatase, or can be subsequently linked thereto. In either case, subsequent contact with a suitable substrate for the enzyme (H_2O_2, glucose, phenylphosphate) gives a reaction for electrochemical measurement either via a ferrocene mediator compound (H_2O_2, glucose) or by oxidation at an electrode surface (phenol from phenyl phosphate). This reaction eventually relates back very sensitively to the presence or amount of initial hybridisation; attomole quantities are measurable.

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Title: NUCLEIC ACID SEQUENCES ATTACHED TO MATERIALS SENSITIVE TO MAGNETIC FIELDS, AND METHODS OF ASSAY AND APPARATUS USING SUCH ATTACHED SEQUENCES.

The present invention is concerned with nucleic acid sequences, assays for nucleic acids and apparatus for such assay.

The assay procedures of the present invention extend to both to detection of the presence of nucleic acids and to the identification of specific nucleic acid molecules, or classes of molecules, by identifying sequences therein, both procedures being needed in many areas of biochemical research and commerce.

Hybridisation between two single-stranded DNA (or RNA) molecules, which have complementary (or partially complementary) sequences has been one approach to the problem of nucleic acid assay. Molecules for use in such assays are capable of binding to a DNA (or RNA) sequence defined to a particular level of sequence homology, and are known as "DNA probes"(or "RNA probes")

Known DNA (RNA) probe techniques all have the

characteristic that the DNA (RNA) polymer formed by hydridisation between the probe and target sequence is not readily detectable by its inherent biochemical activity. It is therefore necessary to mark the polymer formed with some signal-producing chemical or biochemical species.

One particular known method of marking is commonly known as the "Avidin-Biotin Reaction" and relies on the affinity of the egg-white glycoprotein avidin for biotin.

In this method biotin (Vitamin H) is chemically linked to one or more nucleotides, i.e residues which comprise the monomeric units of a DNA polymer. (For convenience of description reference below to DNA probes should be taken to refer also to RNA probes). A single-stranded sequence of a DNA is built up using known units in a known sequence, at least one such being biotin-linked. The sequence still undergoes the classical hybridisation reaction with a complementary strand of DNA to form a double-stranded DNA. It can thus be incorporated into synthetic DNA probes.

In the majority of DNA probe assays, any remaining unbound probe must be separated from the bound probe . This separation is normally done by performing the

hybridisation reaction in conditions under which sample DNA including the target sequence is immobilized on a substrate and following the binding reaction by washing, or centrifugation to remove any excess probe. The bound probe is therefore all that remains, as an indication or measure of the target sequence. It is detected by the addition of avidin, or streptavidin, to which a marker has been attached.

Typically, the marker is an enzyme and is for example horse radish peroxidase or alkaline phosphatase. The marker enzymeis then "developed" by reaction with a substrate which produces a coloured, insoluble precipitate on the membrane surface; the extent of the marker enzyme reaction is usually estimated by eye, or more rarely by densitometry, and although this method provides sensitivity down to the picogram level (Leary et al Proc. Natl. Acad. Sci. USA 80 4045-4049.1983, Chan et al, Nucleic Acids Research 10 8083-8091) it is inherently insensitive and fails to distinguish between single and double copy gene levels.

Our present invention, however, involves a variant on such method of marking the probe sequence, which can also utilise the technology described in our copending European Patent Application 82305597, relating to

methods of assay in which a mediator compound is used to transfer charge from an enzyme to an electrode. Such a system as therein described may be employed to detect the concentration of either a substrate or an enzyme in a sample, by electrochemical measurement of the occurrence or extent of an enzyme-affected reaction. Moreover,our copending European Patent Application 84.303090 discloses a novel route to the measurement of nucleic acid probe sequence coupling by using a method developed from the above in which at least one of a mediator and an enzyme, for use in such electrochemical assay are chemically linked to a nucleic acid probe sequence and where any specific binding, of the probe sequence, to the target sequence which may take place thus affects the electrochemical availability of the chemically linked species, for detection by a sensor electrode in presence of the enzyme substrate. Such technology is also relevant to a consideration of the present invention.

~~One~~ aim of the DNA probe technology and the enzyme detection/assay technology so far developed, has been to detect "inbuilt errors of metabolism" which lead to a variety of "genetic diseases" and inheritable disorders. Among such disorders are: Familial Goiter (iodotyrosine dehalogenase defective), Maple syrup urine disease (alpha-keto decarboxylase defective),

Xanthinuria (Xanthine oxidase defective) and Methaemoglobinemia (Methaemoglobin reductase defective).

A full list of over 3500 conditions due to defective genes can be found in McKusick's "Mendelian Inheritance in Man".

Clearly, in the detection of simple mutations it is necessary to select and recognise relatively short and specific nucleic acid sequences from the entire genetic library of the organism in question. In general, therefore, and in the methods of the prior art mentioned above, a binding reaction must be followed by a recognition step in which the hybridisation event is detected and occasionally quantified.

It will be noted from the above description that there is a requirement for separation of various components of the assay mixture at some stage in the method. One requirement for example is the separation of unbound probe DNA from the probe/target conjugate. This separation requirement has tended to make heterogeneous assays either slow, cumbersome and expensive and has needed skilled technicians. Furthermore, although we have developed assays e.g.using the mediator/redox

systems described in general terms above, a separation and/or concentration step increases the resolution of these assays; there is still a preference if not a need for separation.

The present invention sets out to provide materials, techniques and apparatus to improve the ease of separation in such assays.

According to one aspect of the present invention there is provided an entity including a single or double-stranded nucleic acid sequence linked to a magnetic or magnetisable substance.

Usually, the invention is concerned with a single-strand sequence linked to a magnetic or magnetisable substance, the sequence being a DNA or RNA sequence in a pure or impure form, as obtainable from a natural or a synthetic source. Pure and known sequences will generally comprise a "probe" sequence, but of course a target sequence could be (and usually is) linked to the magnetic or magnetisable substance in accordance with the present invention. Moreover the hybridised or other double stranded DNA (RNA) magnetic/magnetisable entity can also constitute a feature of the invention.

The magnetic/magnetisable substance is usually provided

in a divided or particulate form.

Typically, in this aspect of the present invention, a magnetic or magnetisable particle is bound to the target nucleic acid sequence.

In a preferred form of the invention a substance having a permanent magnetic moment, i.e. a ferromagnetic material, especially iron particles or ferrite particles or magnetite particles, Fe_3O_4, is treated in such a way that the sequence will subsequently attach thereto.

In some embodiments, this attachment can be effected by simple adsorption or by chemical cross-linking directly to the magnetic material, but a more preferable product has a coating on the magnetic substance of a material to which a nucleic acid material can become permanently attached, for example nitrocellulose,and a nucleic acid attached to this coating .Nucleic acids attached to nitrocellulose are known in the chemical art.

The invention also extends to the use of particles of a magnetic or magnetisable substance, coated with a material capable of attachment to a DNA or RNA single-strand material, in a magnetic separation under the influence of an applied magnetic field for the

purpose of separating (a) such nucleic acids from a mixture of materials or (b) attached nucleic acids hybridised with a nucleic acid containing a complementary sequence from excess of the unattached complementary nucleic acid or (c) such attached and hybridised materials from an excess of an enzyme marker, or of a material convertible to an enzyme marker, reactive therewith.

In another aspect of the present invention there is provided a method of separation of single-stranded nucleic acid materials from a complex mixture containing such materials, in which the mixture is treated with magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and the particles and attached nucleic acid materials are thereafter separated from the other components, at least in part, by a magnetic field. As indicated above the coating may be nitrocellulose, but cellulose itself (which generally provides functional groups which can be readily derivatised allowing stable covalent attachment of nucleic acids) can be used.

In yet another aspect of the present invention there is provided a method for the detection or quantification of the presence of a target sequence of nucleic acid units,

or number of such sequences , in a single-stranded DNA or RNA material or mixture of such materials, by contact with a probe DNA or RNA sequence of a predetermined level of homology to the target sequences, followed by separation of the materials for detection or quantification of the probe/target hybridisation reaction; in which the probe sequence or the target sequence is a single-stranded nucleic acid sequence linked to a magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and in which separation is at least in part effected by a magnetic field.

The present invention is particularly concerned with a method for the detection or quantification of the presence of a target sequence of nucleic acid units, or number of said sequences, in a single-stranded RNA or DNA material, or mixture of such materials, in the presence of a complex liquid mixture containing inter alia such DNA or RNA single-stranded materials, comprising the steps of:

(i) contacting the complex liquid mixture with a magnetic or magnetisable material in the form of particles having a coating to which any nucleic acid single-strand RNA or DNA material becomes permanently attached, and thereafter separating the

magnetic particles from the mixture, at least in part, by a magnetic field,

(ii)contacting the separated DNA/RNA material linked to the magnetic or magnetisable particle with a probe single-stranded DNA or RNA sequence to detect or quantify by a hybridisation reaction the presence of the target sequence on said permanently attached DNA/RNA magnetically separated material, and thereafter separating the magnetic or magnetisable material from unbound probe sequence, at least in part, by a magnetic field,

prior to assay of the probe/target bound species.

The probe may be isolated from an organism, or be chemically synthesised, or be synthesised using a host organism.

Assay of the probe-target bound species can be effected by known methods. For example, the probe can be chemically linked with compound A of a pair of compounds A and B which themselves react with a specific binding reaction thus useful for measuring probe level in the separated magnetic materials. Examples of A and B are antigen/antibody; hormone/receptor; lectin/carbohydrate; or cofactor and enzyme all of which give a known and measurable type of assay for the probe content. A

preferred pair of materials A and B are however biotin and avidin, or possibly biotin and streptavidin; the reaction is well known in this context and measurement methods involving fluorescence or enzyme reactions (of an enzyme linked to the avidin) are well-established.

Nonetheless, we have further established a preferred final measurement method preferably using the biotin/avidin, or biotin/streptavidin specific binding system. The method involves attaching biotin to the probe and a suitable e.g. redox enzyme to the avidin or streptravidin.On addition of suitable substrate a product is formed . The extent or occurrence of this reaction can be measured at an electrode directly or conveyed to an electrode by a suitable mediator such as a metallocene, more especially a ferrocene as disclosed in our earlier Applications itemised above.

The enzyme can be glucose oxidase, or glucose dehydrogenase, for use with glucose as a substrate. The preferred enzyme is however a peroxidase e.g. horseradish peroxidase, for use with H_2O_2 as a substrate. More generally speaking, any redox enzyme can be measured, e.g. in addition to the above, dehydrogenases or those enzymes capable of attacking or degrading large molecules e.g. α-amylase, RNA-ases or DNA-ases.

It is believed however that many of the enzyme-substrate pairs disclosed in the Applications identified above which we have studied could be utilised in association with the mediator in this aspect of the method of the present invention, given some limitations on the assay conditions which would be obvious to the man skilled in the art. One example of such a necessary variation would be if the substrate of one particular enzyme were for some reason present in the assay mixture. Of these possible pairs, it is clearly advantageous to utilise those enzyme/substrate pairs whose behaviour is established in most detail and which give good, and preferably linear, response over the expected measurement range.

Ferrocenes (bis cyclopentadienyl iron and its derivatives) have chemical and electrochemical advantages over other mediators used with enzyme/ substrate reactions for charge-transfer purposes.A number of ferrocene compounds are disclosed in more detail below.

The enzyme of particular preference is glucose oxidase although the enzyme horse radish peroxidase may also be used. The mediator of preference is ferrocene or a derivative thereof. Suitable protocols for the measurement of glucose oxidase concentration by an

electrochemical method in the presence of ferrocene are given in the above referenced patent applications.

As described in the patent applications, identified above, it is possible to detect the presence of oxidoreductases using amperometry or coulometry, by coupling the electron transfer from the substrate to the enzyme and thence to a sensor electrode. Such an electrode is generally referred to as an "electron transfer electrode".

In a specific embodiment of the present invention therefore there is provided a method for detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a celluose derivatives (DCEL) such as nitrocellactose (NITC) whereby single-stranded nucleic acids (NUCA) become permanently attached thereto, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(ii) contacting the (NUCA)-(NITC)-(MAG) material thus separated with a single-stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the

(NUCA)-(DCEL)-(MAG) particles possessing a complementary target sequence will react, with hybridisation to give the complex (BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field

(iii) contacting the separated materials with avidin or streptavidin (AV) linked with a peroxidase enzyme (PER) to form the complex (PER)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field, and

(iv) bringing the said magnetically separated materials carrying the said complex into contact with hydrogen peroxide and a ferrocene, whereby the terminal (PER) group on the complex causes the hydrogen peroxide to react so that the ferrocene transfers charge to or from acontacting electrode to feed a readout signal.

In another variant of this method of measurement the preferred particle coating is cellulose, and the enzyme is an alkaline phosphatase, capable of producing phenol, for oxidation at a working electrode, from a phenyl phosphate substrate. Such an enzyme can be itself linked by biotin to the avidin (or streptavidin) after the biotin-avidin reaction on the hybridised material.

Thus, in one form of the invention there is provided a method for the detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a derivatised cellulose (DCEL) capable of permanently attaching thereto single-stranded nucleic acids, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(ii) contacting the (NUCA)-(DCEL)-(MAG) material thus separated with a single stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the (NUCA)-(DCEL)-(MAG) particles possessing a complementary target sequence will react with hybridisation to give the complex (BIO)-(PRO)-(NUCA)-(DCEL)-MAG, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iii) contacting the separated materials with avidin or streptavidin (AV) to form the complex (AV)-(BIO)-(PRO)-(NUCA)-DCEL)-(MAG) and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iv) thereafter linking to the (AV)-groups of the

complex a marker enzyme (ENZ), and

(v) bringing the resultant (ENC)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG into contact with a suitable substrate for the said enzyme and measuring the enzyme reaction electrochemically.

The cellulose derivative (DCEL) can be an aminophenylthioether linkage, activated to the corresponding diazo compound for immobilisation of the single-strand (NUCA).

The (AV) group can be itself - biotin-linked to the further enzyme, which in one preferred embodiment is an alkaline phosphatase utilising phenyl phosphate as a substrate convertible to phenol, or other substrates. In such a case direct, unmediated, measurement is possible.

The invention further extends to apparatus for use in the method of assay as defined above comprising an upper vessel, a lower vessel selectively placeable in liquid flow communication therewith, and selectively applicable magnetic element in said upper vessel. Possibly the magnetic element is a magnetisable, or magnetic, grid element having two layers relatively movable to provide either separation or communication between the vessels.

Alternatively, the magnetic element may be an electrode for subsequent use in the electrochemical determination of the enzyme reaction; if so it has the potential advantage of drawing towards itself the magnetic species.

According to the invention therefore, if separation procedures (including washing procedures) are required, use may be made of the magnetic properties of the various complexes either by placing tubes containing the complexes in a magnetic or magnetisable test-tube rack or by making use of a preferred apparatus disclosed below. In this manner substances which have not become linked to the coated magnetic particles may be removed from the assay sample. Thus the target DNA or RNA may be separated from debris such as other broken-cell products, and buffer or other solutions.

The invention will be further described by way of example and with reference to the accompanying drawings, wherein;

Figure 1 diagrammatically illustrates steps of an assay method according to the present invention,

Figures 2a and 2b show features of an apparatus according to the present invention,generally in vertical

cross-section.

Figure 3, in its three component parts, shows diagrammatically steps in a method of assay of the present invention carried out in apparatus according to the present invention.

Figure 4, in its three component parts, shows variant electrode configurations which can be employed in the method of assay of the present invention, and

Figure 5 is a graph of concentration in attomoles of λ -DNA, immobilised by hybridisation with a target DNA attached to a cellulose-coated magnetic particulate substrate and labelled with an alkaline phosphatase to produce phenol from a phenyl phosphatase substrate against current in micro-amps at a pyrolytic graphite electrode.

Example 1

Figure 1, step 1, shows the addition of a nitrocellulose-treated magnetic particles (MAG)(NITC) to a mixture containing single stranded nucleic acids (NUCA) to form a complex (MAG)-(NITC)-(NUCA).

It will be understood that any nucleic-acid-linkable cycling material can be used, and that cellulosic materials, of which nitrocellulose is only a non-limiting examples, are preferred but not essential. An optional magnetic separation can now take place (as described in more detail below) whereby non-DNA or non-RNA material can be separated from the mixture. This leaves a magnetic particle complex of various nucleic acids to be investigated for the target sequence.

Step 2 shows addition of excess of a probe sequence linked to biotin, which by a hybridisation reaction forms the complex (MAG)-(NITC)-(NUCA)-(PRO)-(BIO) with those nucleic acids containing the target sequence, leaving excess (PRO) - (BIO) unreacted. A magnetic field is applied to retain all complexes containing the (MAG) and therefore those complexes including the target nucleic acid. The excess (PRO)-(BIO) can therefore be washed away without substantial loss of the complex of probe and target nucleic acid. The other (NUCA) species are still present, but not labelled with (PRO)-(BIO).

Step 3, shows addition of excess of a detector system for the biotin marker employed. The detector system in this particle example comprises a conjugate of avidin or streptavidin (AV) and horseradish peroxidase (PER), although other systems can of course be used. Upon

addition of this conjugate to the sample containing the biotinylated probe there is a binding reaction between the avidin and the biotin to link the peroxidase to the target and probe nucleic acid complexes only, i.e. not all nucleic acids present;

Thus the system comprises (MAG)-(NITC)-(NUCA)-(PRO)-(BIO)-(AV)-(PER) plus excess (AV)-(PER).
After allowing time for the reaction to proceed, a magnetic field is applied to retain the large complex and the excess (AV)-(PER) is washed away without disturbing the large complex.

At this stage the assay mixture will still contain much or all of the original nucleic acid which was absorbed onto the coated magnetic particles. However, only those target sequences which are homologous to the probe have formed a duplex with the probe and have therefore complexed the peroxidase. Hence, the concentration of peroxidase in the solution is related directly to the degree of duplex formation and therefore to the degree of homology between the original target nucleic acid and the probe.

Step 4 shows an electrode (E) placed in contact with the resulting suspension containing (MAG)-(NITC)-(NUCA)-(PRO)-(BIO)-(AV)-(PER). The

H_2O_2, the substrate of the peroxidase, is added together with ferrocene as a suitable mediator compound. The catalytic current generated is proportional to the (AV)-(PER) activity and hence to the concentration of the nucleic acid which has been hybridised to the probe.

At this stage it is possible therefore to obtain a measurement of the target nucleic acid concentration. Furthermore, as the assay mixture still contains much of the original nucleic acid (in a partially purified form) it is possible to probe the assay mixture again for other sequences, by the use of other probes. Unless these additional probes bind to sequences which overlap with those which have already formed a duplex, further hybridisation can occur between the target nucleic acid and the new probe and the analysis may be continued (with a suitable compensation for the background peroxidase activity). As the concentration of the peroxidase can only be represented at the electrode in the presence of peroxide, further probes which employ enzymes different from peroxidase, and therefore show charge transfer in the presence of different substrates, are more usefully employed in the detection of other sequences in the same assay mixture.

Figure 2 shows one form of an apparatus according to the

present invention and for use in the method of the present invention. It consists of two vessels (1 and 2) separated by a magnetic filter (3). Flanged edges (4 and 5) are provided to seal the joint between the vessels. The filter comprises a movable magnetic or magnetisable grid or gauze (6) together with a fixed gauze (7) which latter for ease of operation should preferably be coated with a teflon$_{TM}$ or teflon$_{TM}$-like material. The magnetic gauze (6) can if necessary be used as an electrode; if so it should have a surface of gold or other suitable metal, electroplated or otherwise deposited onto a magnetisable or magnetic gauze support. If the grid or gauze is not to be used as an electrode, it can be coated with a material such as teflon$_{TM}$ and silanised to inhibit absorption. The interior of the vessels should also be silanised before use or made of a suitable material such as polypropylene to prevent absorbtion of materials on to the vessel walls.

~~The~~ lower vessel (2) is provided with a drain (16) and a suction port (15).

The grid or gauze can be constructed of any permanent magnetic material, such as iron, cobalt or nickel. Alternatively, if windings are placed around the joint, a potentially magnetisable material can become magnetic

on the passage of a current and demagnetised when the current is off.

The flanged edges (4 and 5) may be greased, or provided with O-rings (9) or both. Alternatively, a bayonet-type fitting may be employed. An exterior holding clip or clamp (8) may be provided to ensure a good seal and/or rigidity of the apparatus.

In the embodiment shown in Fig. 2, the upper grid (6) is movable relative to the lower grid by a sliding action. In the figure, the upper grid is shown to be moved by a tag (10). A number of other methods can be used to provide movement such as a rod along the axis of the upper vessel. The tag, however is advantageous in that it may form an electrical terminal if the upper grid (6) is to constitute an electrode. When the upper grid (6) moves relative to the lower materials can be caused to fall from the upper chamber to the lower chamber and be removed from the assay mixture.

Further detail of the sliding action is shown in Figures 2a and 2b. The two grids are open to the passage of materials in Figure 2a but closed to the passage of materials in Figure 2b.

A coil (11) is located about the lower part of the upper vessel and generates a magnetic field when current is

passed through it. (As an alternative, a permanent magnet may be employed, either hand-held or as part of a test-tube rack).

Fig. 3 shows steps of a method of the present invention carried out in an apparatus according to the present invention.

In the first portion of the figure there is shown a vessel in which (MAG)-(NITC)-(NUCA) (by way of example) has already been formed and separated from an earlier mixture or else in which a pure nucleic acid sample is utilised not needing such separation. The biotinylated probe (PRO)-(BIO) is added, in suitable excess. Time is allowed for duplex formation to occur between the (PRO)-(BIO) probe and the target sequences in the (MAG)-(NITC)-(NUCA). During this stage of the reaction, the composite grid 3 i.e. the two grids or gauzes 6 and 7 are closed to the passage of materials from the upper vessel (1) to the lower vessel (2).

In the second drawing of Fig. 3, to remove the excess (PRO)-(BIO) from the assay mixture a magnetic field is applied to the apparatus by the coil (11). Ferromagnetic members (13) may if desired be placed within the field generated by the coil (11) so that the magnetic particles (12) become immobilised upon them and

are held in the upper vessel (11). Alternatively, if a magnetic or magnetisable grid is present the magnetic particles (12) attach themselves to this grid when the field is on.

The grid is then opened to the passage of materials from the upper vessel to the lower vessel. The assay mixture may therefore be washed through by the addition of a suitable fluid from the direction indicated by the letter A in the figure. Excess (PRO)-(BIO) therefore becomes washed out of the apparatus in the direction indicated by the letter B.

In the third portion of Fig 3 the grid (3) is closed again and a suitable buffer solution is placed in the upper chamber (1). The current to the coil (11) (or grid) is switched off. The magnetic particles return into suspension or solution within the upper chamber.

The above description relates to removal of excess (PRO)-(BIO) from the assay mixture. However, other magnetic separation stages i.e. the previous (NUCA)-(NITC)-(MAG) separation or the subsequent separation in which excess detector is removed from the assay mixture, has a like formal protocol.

The coil (11) may if desired also be employed to agitate

the magnetic particles within the upper chamber by the application of a suitably varying magnetic field.

Fig. 4 shows in its three parts and in diagrammatic cross-sections three different electrode configurations suitable for use with the apparatus of the present invention in the final stage of direct electrochemical measurement of the enzyme reaction.

Fig. 4a shows a more or less orthodox three-electrode configuration comprising a working electrode (W), an auxiliary electrode (A) and a reference electrode (R). Further details and examples of electrode configurations of this general type and of a similar, two-electrode, type are given in the patent applications identified above.

Fig. 4b shows a working electrode (W) provided with a winding (14) to enable the electrode itself to become magnetic as and when desired. This has the effect of concentrating on the electrode reagents which are per se magnetic or magnetisable.

Fig. 4c shows the working electrode (W) constitute as one or other component of the grid.

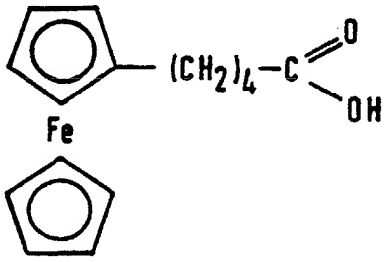
The following example shows stages in the preparation

and properties of a particularly valuable ferrocene derivative which can be utilised in one preferred electrochemical measurement procedure, and which exemplify another aspect of the present invention which consists in a nucleotide labelled with a covalently-linked electoactive species, e.g. a metallocene such as ferrocene; more specifically a ferrocene-labelled -UTP is envisaged. Another aspect of the invention comprises the use of such materials in the assay of nucleic acid sequences.

Example 2

PART I

Preparation of 5-Ferrocenyl Pentanoic Acid



5-Ferrocenyl pentanoic acid was prepared by the procedure published in J.Am. Chem. Soc. (1957) 79, 3420 by Rinehart et al.

PART II

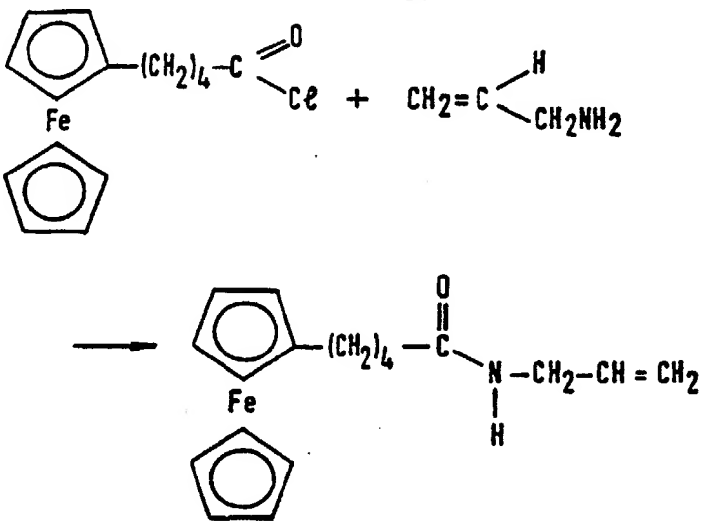
Preparation of N-Propen-2-enyl.5-Ferrocene Pentamide:

$Fc\ (CH_2)_4CONHCH_2CH{=}CH_2$

(i) Preparation of Ferrocenyl Pentanoyl Chloride

$Fc\ (CH_2)_4COCl$

To a solution of 5-Ferrocenyl pentanoic acid in Toluene (500ml) containing pyridine (1.0mL) under dinitrogen atmosphere was added, dropwise, a solution of phosphorous trichloride (PCl_3:2.87 g (1.83mL) 21 mmol). The solution turned cloudy with the formation of a fluffy white precipitate. After completing the addition, the mixture was refluxed for 4 hours. After cooling to ambient temperature, the yellow solution was decanted and the volatiles removed under reduced pressure to give an orange oil. This oil was dissolved in hexane and used in the next step without further purification.



To a vigorously stirred solution of allylamine (1.6 g (2.1mL; 28 mmol) in freshly dried and distilled Tetrahydrofuran (THF) (100 mL) under a nitrogen atmosphere was added dropwise, over a period of 15 minutes, the hexane solution of $Fc(CH_2)_4COCl$. After stirring for 30 minutes the solution was filtered to remove an offwhite precipitate. Removal of volatiles under vacuum gave the title compound as a yellow powdery solid.

Yield 3.9 g (86%). M.Pt 76-78°C.

Mass spectrum: $M.^+/e$ 325 for $FeC_{18}H_{23}NO$

I.R. (Nujol; KBr plates) 3195 cm^{-1}(N-H);1640 cm (C=O); 1550 cm^{-1}(N-H);1110 and 1005 cm^{-1}(C-H) for the ferrocene ring

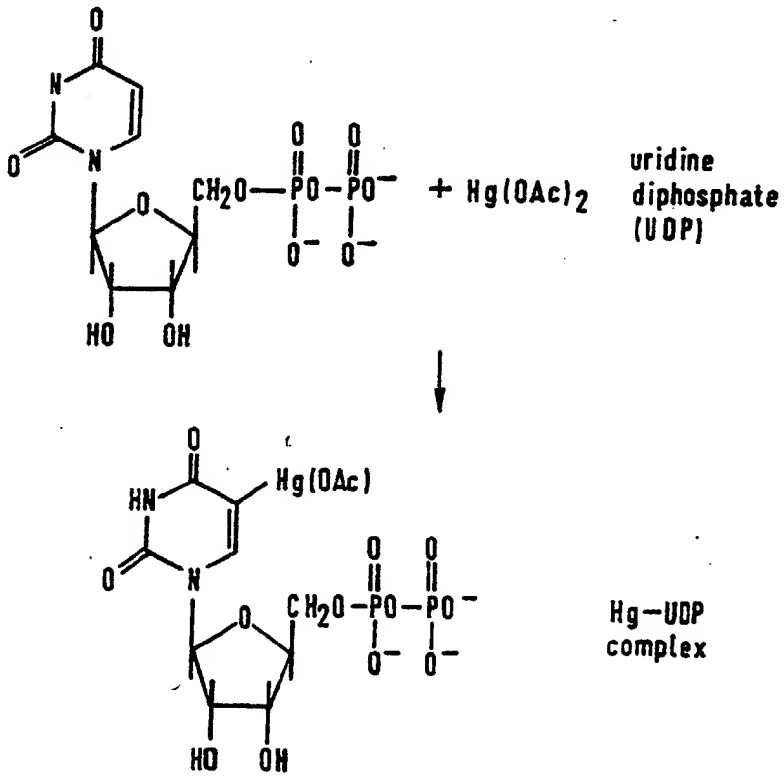
^{1}HNMR($CDCl_3$);5.85(m,1H,C<u>H</u>=CH_2);5.44(S,br,1H,NH) 5.17(m,2H,CH=C<u>H</u>$_2$);4.10(S,5H,C_5H_5);4.05(m,4H,C_5H_4); 3.89(m,2H,allyl C<u>H</u>$_2$);2.36(t,2H,CH_2);2.21(t,2H,CH_2) 1.69(q,2H,CH_2) and 1.53(q,2H,CH_2).

PART III

Preparation of Ferrocene-Uridine Diphosphate Conjugate:

$Fc(CH^2)_4CONH\ CH_2CH{=}CH{=}UDP$

i) Mercuration of Uridine Diphosphate at C-5



The uridine diphosphate (UDP) (Sigma) was mercurated at the C-5 position by the literature procedure described by Langer et al Proc. Natl. Acad.Sci. USA (1981),78,6633.

(ii)Preparation of Ferrocenylated - UDP

$$Hg\text{-}UDP + Fc(CH_2)_4CO\cdot NH\cdot CH_2CH=CH_2 \xrightarrow{K_2PdCl_4} Fc(CH_2)_4CO\cdot NH\cdot CH_2CH=CH\text{-}UDP$$

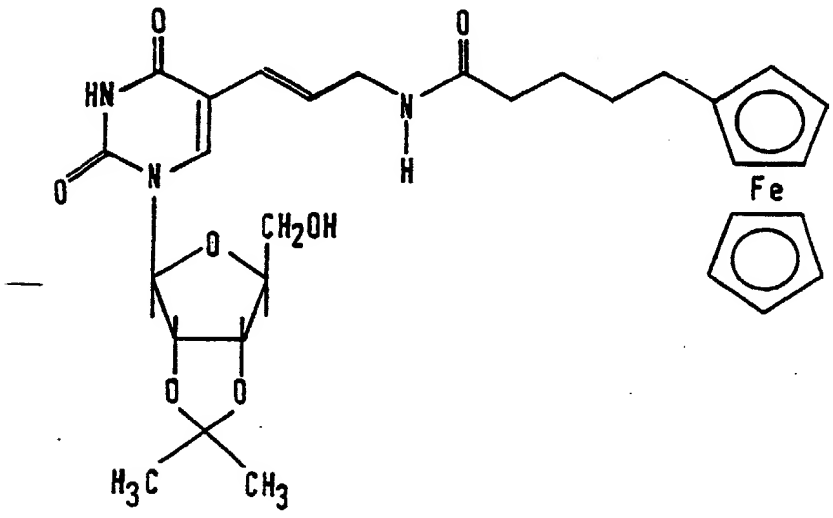
To a solution of mercurated UDP (380 mg; 0.56 mmol) dissolved in 0.1 M sodium acetate (100 mL; pH 5.0) was added a solution of $Fc(CH_2)_4CONHCH_2CH=CH_2$ (360 mg; 1.12 mmol) in tetrahydrofuran (25mL). Addition of potassium tetrachloropalladate, K_2PdCl_4, (ALdrich); 183 mg (0.56 mmol) dissolved in deionized water (15mL) resulted in an instantaneous formation of a black powder. The mixture was stirred at ambient temperature for 3 days. The black precipitate was removed by centrifugation to give a blue-green supernatant solution which was decanted. The precipitate was washed with further sodium acetate (0.1M; 2x25mL). To the combined sodium acetate solution was added three times by volume of ethanol which on cooling to -20°C gave a fine brown precipitate which was collected by centrifugation and

removal of the supernatant and washed successively with with ethanol and diethyl ether and dried (yield; 100 mg).

IR(KBr disc): 3420 cm^{-1} (υO-H) 1680 cm^{-1}(υC=O); 1550cm^{-1}(σN-H);1240cm^{-1}(λP=O);1060$cm^{-1}\lambda$P=O).
Due to the broadness of the above bands, absorptions due to the ferrocenyl group are obscured. However, cyclic voltametric studies confirm the presence of the ferrocene moiety.

PART IV

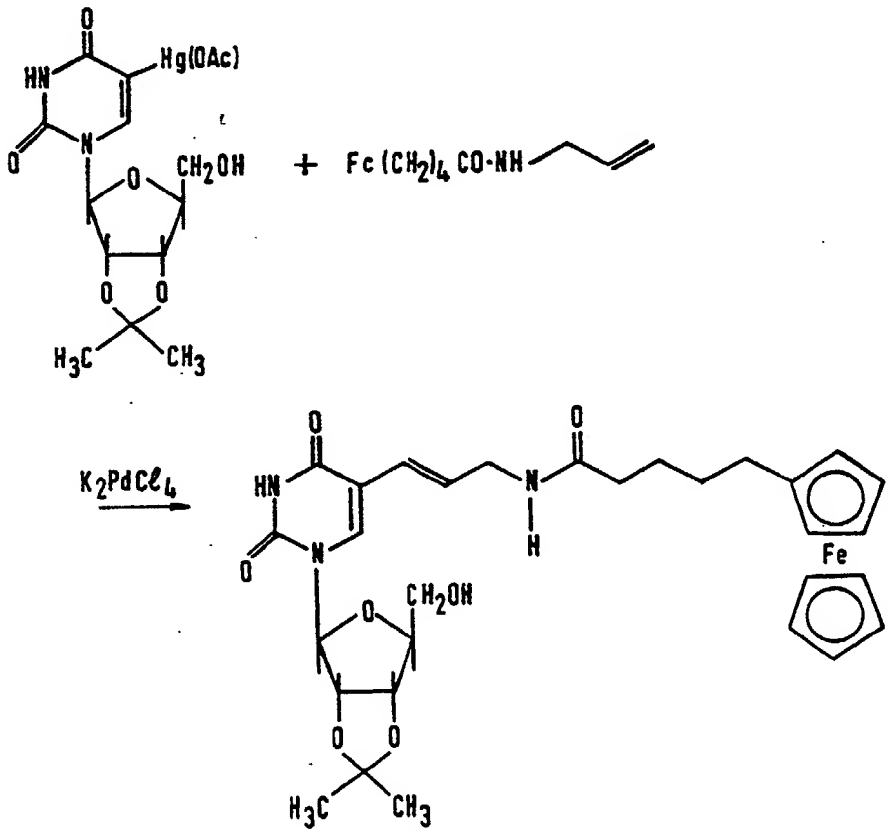
Preparation of 2',3'Isopropylidene Uridine-Ferrocene Conjugate



SUBSTITUTE SHEET

(i) Mercuration of 2',3' Isopropylidene Uridine

To a solution of 2',3' isopropylidene uridine (2.0g; 8.06 mmol) in deionised water (400 mL) was added mercuric acetate (12.84g; 40.28 mmol). The solution was heated at 50°C for 4 hours and cooled in an ice bath. Lithium chloride (1.40 g; 32.22 mmol) was added and the solution extracted with ethylacetate (6x200mL) to remove excess mercuric chloride. The aqueous solution was used in the subsequent step without further purification.



To a solution of mercurated isopropylidene uridine prepared above was added; a solution of $Fc(CH_2)_4CONHCH_2CH=CH_2$ (3.17 g;9.8 mmol) dissolved in THF (200 mL). Addition of a solution of K_2PdCl_4 (263 mg 8.1 mmol) in deionised water (25mL) resulted in an instantaneous formation of a very fine black precipitate. The reaction mixture was stirred for 17 hours and filtered to remove the precipitate.To the green filtrate was added L.Ascorbic acid (1.55 g) together with diethylether (500mL). The mixture was vigorously shaken and the organic layer separated from the colourless aqueous layer. The ether phase was dried over magnesium sulphate and removal of the volatiles gave an orange oil.

The product was further purified by column chromatography on silica with acetone-ether (1:1) as eluant. The desired compound eluted as a major yellow band after two minor yellow components and forms an orange oil on removal of volatiles, which subsequently crystallised to a yellow solid on standing at room temperature. This was identified as ferrocenylated 2'-3' isopropylidene uridine conjugate.

Melting point 60-62°C

I.R.(KBr disc) 3300 cm^{-1}(νO-H); 3090 cm^{-1}(μ N-H)
1725 and 1640 cm^{-1} (ν C=O);1550 cm^{1} (σ N-H);
1110 and 1005 cm^{-1}.δ(C-H ferrocene)

^{1}H NMR ($CDCl_3$)

σ9.80 (S,1H,NH); 5.87 (S,1H,NH); 6.16 (m,1H); 5.31 (S,1H,H-6); 4.17 (d,1H); 4.09 (S,5H,C_5H_5); 4.05 (m,4H,C_5H_4); 3.54 (,2H); 2.74 (t,2H,CH_2); 2.4-2.2 (m); 2.21 (S,3H,Me); 2.14 (t,2H,CH^2); 1.66(m,2H,CH_2) 1.62(s,3H,Me;1.50(m,2H,CH_2);0.87(m,1H)

Example 3

This example shows a variant procedure and use of a different electrochemical measurement system.

Materials

All chemicals, unless specifically stated otherwise, were obtained from BDH Ltd. Streptavidin and alkaline phosphatase were obtained from BRL Ltd.

Methods

(a) Cellulose-coated magnetic particles were prepared as described in the literature (Pourfarzaneh et al, Methods Biochem Anal. 28, 267-295 1982.

(b) Magnetic particles were derivatised by reaction with 1,4-butanediol diglycidyl ether/2-aminothiophenol (APTE)as described by Bunemann et al Nucleic Acids Research 10 7163-7180. 1982.

(c) The APTE-matrix was activated by conversion to the corresponding diazophenylthioether (DPTE)-matrix as described by Bunemann et al (see above). The diazo-matrix was prepared immediately prior to the immobilisation of DNA.

(d) Coupling of denatured DNA (as a target material) to the DPTE-support was achieved in the following manner. λ DNA was suspended in 1xSSPE buffer and denatured by the addition of DMSO to produce an 80% v/v mixture of DMSO in buffer. Sonicated salmon sperm DNA was added as carrier to produce a final concentration of 1 mg/ml. The DNA solution (1 ml) was then added to the wet DPTE material (2 g) and processed as described by Bunemann et al, see above

(e) λDNA was biotinylated (to provide a probe material) using nick-translation kit (purchased from BRL Ltd) in the presence of biotin-11-UTP. High molecular weight DNA was purified from unincorporated material by chromatography on a Nensorb cartridge (Dupont). The DNA solution was evaporated to dryness and the pellet was

reconstituted in 1xSSPE buffer at 37° for 20 min.

(f) Hybridisations were carried out in polypropylene vials, sealed in polypropylene bags, submerged in a shaking water bath. λDNA samples linked to the magnetic support were pre-incubated at 65°C for two hours in a cocktail which consisted of 4 x SSPE, 6 x Denhardt's solution, 300µg/ml denatured salmon DNA and 0.1% w/v SDS (1 ml).

The pre-hybridisation mix was removed and replaced with 1 ml of hybridisation cocktail consisting of 4 x SSPE, 2 x Denhardt's solution, 200 µg/ml denatured salmon sperm DNA, 0.1% w/v SDS, 10% w/v dextran sulphate in the following buffers:

(1) 2 ml of 2 x SSC (3 x 5 min at 20°C)

(2) 2 ml of 0.2 x SSC (3 x 15 min at 65°C)

Unbound probe was removed by attraction of the magnetic particles using equipment as shown above, and suitable washing procedure.

(g) Detection of biotinylated probes

Binding of streptavidin and poly alkaline phosphatase was conducted essentially as described in the dot-blot procedure of Chan et al (1985) see above.

(1) Firstly, the magnetic particles were washed at

22°C for 90 min in 1 ml blocking buffer (100 mM NaCl, 100 mM Tris/HCl, 3 mM $MgCl_2$, 0.5% $^{v}/v$ Tween 20, at pH 7.5), magnetic separation as before also being utilised as a separation technique.

(2) The blocking buffer was replaced with 1 ml of a solution of streptavidin (2 µg/ml in above buffer, except that Tween 20 was at 0.05% $^{v}/v$); the incubation was for 10 min at 22°C.

(3) The support was washed 3 x (15 min each) with blocking buffer and finally resuspended in 1 ml of biotinylated poly alkaline phosphatase solution (1 µg/ml in 0.05% $^{v}/v$, Tween 20/blocking buffer). Incubation was for 10 min at 22°C. Thus, the enzyme became attached, via biotin, to the streptavidin, itself biotin-linked to the hybridisation product.

(4) The matrix was then washed to remove unbound phosphatase, in the following manner; 3 x 1.5 ml blocking buffer (5 min each at 22°C), followed by 2 x 1.5 ml developing buffer (10 min each at 22°C), the magnetic procedures again being utilised to retain the desired material. Developing buffer consisted of 100 mM NaCl, 160 mM Tris/HCl, 10 mM $MgCl_2$, pH 9.6.

(5) Alkaline phosphatase bound to the magnetic

matrix was finally detected electrochemically by resuspending the supporting material in 1 ml of developing buffer which contained 5 mM phenyl phosphate, and incubating at 22°C for 10 min. The supernatant was decanted and transferred to a standard three electrode cell (saturated calomel reference, platinum counter and pyrolytic graphite working electrode). Phenol produced by the action of alkaline phosphatase was oxidised at the working electrode which was poised at +600 mV, and the resulting current versus time transients were recorded.

Figure 5 shows the currents obtained when magnetic particles containing various amount of immobilised DNA were probed using the protocols described in the methods section. The results demonstrate the sensitivity of the detection system employed in that 1 attomole of DNA was readily detected above background levels. Furthermore, in the range examined, the current response is directly proportional to the amount of immobilised DNA on the magnetic support. The simple one step hybridisation protocol described above thus has the potential to detect DNA sequences present at the single copy level in mammalian DNA and to allow the quantitation of multiple copy gene sequences. The system can be extended and modified to produce further configurations, for example;

(a) Any marker enzymes that can be either biotinylated and/or covalently linked to streptavidin can be used provided that they produce electrochemically active products (i.e. glucose oxidase, horse radish peroxidase).

(b) The immobilisation of target DNA onto the magnetic support could be circumvented by using a 'sandwich' type procedure, as described previously in the literature by Ranki et al Gene 21, 77-85 (1983) and more recently by Langdale and Malcolm, Gene 36, 201-210 (1985) in which two non-overlapping restriction fragments of the gene of interest are utilised. Fragment A is covalently linked to the magnetic support and fragment B is labelled with biotin. The presence of a contiguous DNA/RNA sequence in a crude DNA mixture that is capable of hybridising to both A and B will effectively result in the biotinylation of the magnetic support (detected as described above).

(c) The ease of handling of the magnetic solid phase support and the convenience and speed of the washing steps facilitated by its paramagnetic nature mean that the system could readily be incorporated into an automated analytical device.

CLAIMS

1. An entity including a single or double-stranded nucleic acid sequence linked to a magnetic or magnetisable substance.

2. An entity as claimed in Claim 1 in which the nucleic acid is single-stranded.

3. An entity as claimed in Claim 2 provided in particulate form.

4. An entity as claimed in Claim 3 in which the nucleic acid is of unknown composition for investigation as containing a target sequence.

5. An entity as claimed in Claim 3 in which the particles are ferromagnetic.

6. An entity as claimed in Claim 5 in which the particles are coated with a material to which the nucleic acid attaches.

7. An entity as claimed in Claim 6 in which the coating material is a cellulose derivative.

8. An entity as claimed in Claim 7 in which the

derivative is nitro-cellulose.

9. The use of particles of a magnetic or magnetisable substance, coated with a material capable of attachment to a DNA or RNA single-strand material, in a magnetic separation under the influence of an applied magnetic field for the purpose of separating (a) such nucleic acids from a mixture of materials or (b) attached nucleic acids hybridised with a nucleic acid containing a complementary sequence from excess of the unattached complementary nucleic acid or (c) such attached and hybridised materials from an excess of an enzyme marker, or of a material convertible to an enzyme marker, reactive therewith.

10. A method of separation of single-stranded nucleic acid materials from a complex mixture containing such materials, in which the mixture is treated with magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and the particles and attached nucleic acid materials are thereafter separated from the other components, at least in part, by a magnetic field.

11. A method for the detection or quantification of the presence of a target sequence of nucleic acid units

or number of such sequences , in a single-stranded DNA or RNA material or mixture of such materials, by contact with a probe DNA or RNA sequence of a predetermined level of homology to the target sequence, followed by separation of the materials for detection or quantification of the probe/target hybridisation reaction; in which the probe sequence or the target sequence is a single-stranded nucleic acid sequence linked to a magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and in which separation is at least in part effected by a magnetic field.

12. A method for the detection or quantification of the presence of a target sequence of nucleic acid units, or number of said sequence, in a single-stranded RNA or DNA material, or mixture of such materials, in the presence of a complex liquid mixture containing inter alia such DNA or RNA single-stranded materials, comprising the steps of:

(i) contacting the complex liquid mixture with a magnetic or magnetisable material in the form of particles having a coating to which any nucleic acid single-strand RNA or DNA material becomes permanently attached, and thereafter separating the magnetic particles from the mixture, at least in

part, by a magnetic field,

(ii)contacting the separated DNA/RNA material linked to the magnetic or magnetisable particle with a probe single-stranded DNA or RNA sequence to detect or quantify by a hybridisation reaction the presence of the target sequence on said permanently attached DNA/RNA magnetically separated material, and thereafter separating the magnetic or magnetisable material from unbound probe sequence, at least in part, by a magnetic field,

prior to assay of the probe/target bound species.

13. A method as claimed in Claim 10, 11 or 12 in which the coating is a cellulose derivative.

14. A method as claimed in Claim 10, 11 or 12 in which the coating is nitro-cellulose.

15. A method as claimed in Claim 12 in which assay is effected by linkage of the probe sequence with compound A of a pair of compounds A and B which themselves react with a specific binding reaction, whereby B can be incorporated into the bound probe and utilised as a marker, or to attach a marker, for subsequent assay.

16. A method as claimed in Claim 15 wherein compound A

is biotin and compound B is avidin or streptavidin linked to an enzyme the effect of which on a substrate is used for the subsequent assay.

17. A method as claimed in Claim 16 in which the enzyme action is detected electrochemically and the enzyme is any redox enzyme more particularly a dehydrogenase, or a enzyme which degrades a large molecule such as α-amylase, an RNA-ase or a DNA-ase.

18. A method as claimed in Claim 17 in which the electrochemical activity of the enzyme is detected with use of a mediator compound.

19. A method as claimed in Claim 18 in which the mediator is a metallocene, especially a ferrocene.

20. A method as claimed in Claim 19 in which the enzyme/substrate system is peroxidase/H_2O_2 or glucose oxidase/glucose.

21. A method for detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a cellolose

derivative (DCEL) whereby single-stranded nucleic acids (NUCA) become permanently attached thereto, and thereafter separating the magnetic particles from the mixture
by a magnetic field,
(ii) contacting the (NUCA)-(DCEL)-(MAG) material thus separated with a single-stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the (NUCA)-DCEL)-(MAG) particles possessing a complementary target sequence will react, with hybridisation to give the complex
(BIO)(-PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field
(iii) contacting the separated materials with avidin or streptavidin (AV) linked with a peroxidase enzyme (PER) to form the complex
(PER)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field, and
(iv) bringing the said magnetically separated materials carrying the said complex into contact with hydrogen peroxide and a ferrocene, whereby the terminal (PER) group on the complex causes the hydrogen peroxide to react so that the ferrocene transfers charge to or from a contacting electrode

to feed a readout signal.

22. A method for the detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a derivatised cellulose (DCEL) capable of permanently attaching thereto single-stranded nucleic acids, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(ii) contacting the (NUCA)-(DCEL)-(MAG) material thus separated with a single stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the (NUCA)-(DCEL)-(MAG) particles possessing a complementary target sequence will react with hybridisation to give the complex (BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iii) contacting the separated materials with avidin or streptavidin (AV) to form the complex (AV)-(BIO)-(PRO)-(NUCA)-DCEL)-(MAG) and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iv) thereafter binding to the (AV)-groups of the complex using biotin (BIO) a marker enzyme (ENZ), and (v) bringing the resultant (ENZ)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG) into contact with a suitable substrate for the said enzyme and measuring the enzyme reaction electrochemically.

23. A method as claimed in Claim 21 or 22 in which the cellulose derivative (DCEL) is an nitrocellulose or aminophenylthioether linkage, activated to the corresponding diazo compound for immobilisation of the single-strand (NUCA).

24. A method as claimed in Claim 22 in which the (AV) group is itself biotin-linked with an alkaline phosphatase utilising phenyl phosphate as a substrate conversible to phenol.

25. Apparatus for use in a method of assay as defined in Claim 10, 11 or 12 comprising an upper vessel, a lower vessel selectively placeable in liquid flow communication therewith, and aselectively applicable magnetic element in said upper vessel.

26. Apparatus as claimed in Claim 25 in which the magnetic element is a magnetisable or magnetic grid element having two layers relatively movable to provide

either separation or communication.

27. Apparatus as claimed in Claim 25 in which the magnetic element is an electrode for subsequent use in the electrochemical determination of anenzyme reaction.

28.A nucleotide labelled with a covalently-linked electroactive species.

29. A ferrocene-labelled nucleotide.

30. A ferrocene-labelled UTP.

31. The use of a nucleotide material labelled as claimed in claim 28, 29 or 30 in the assay of nucleic acid sequences.

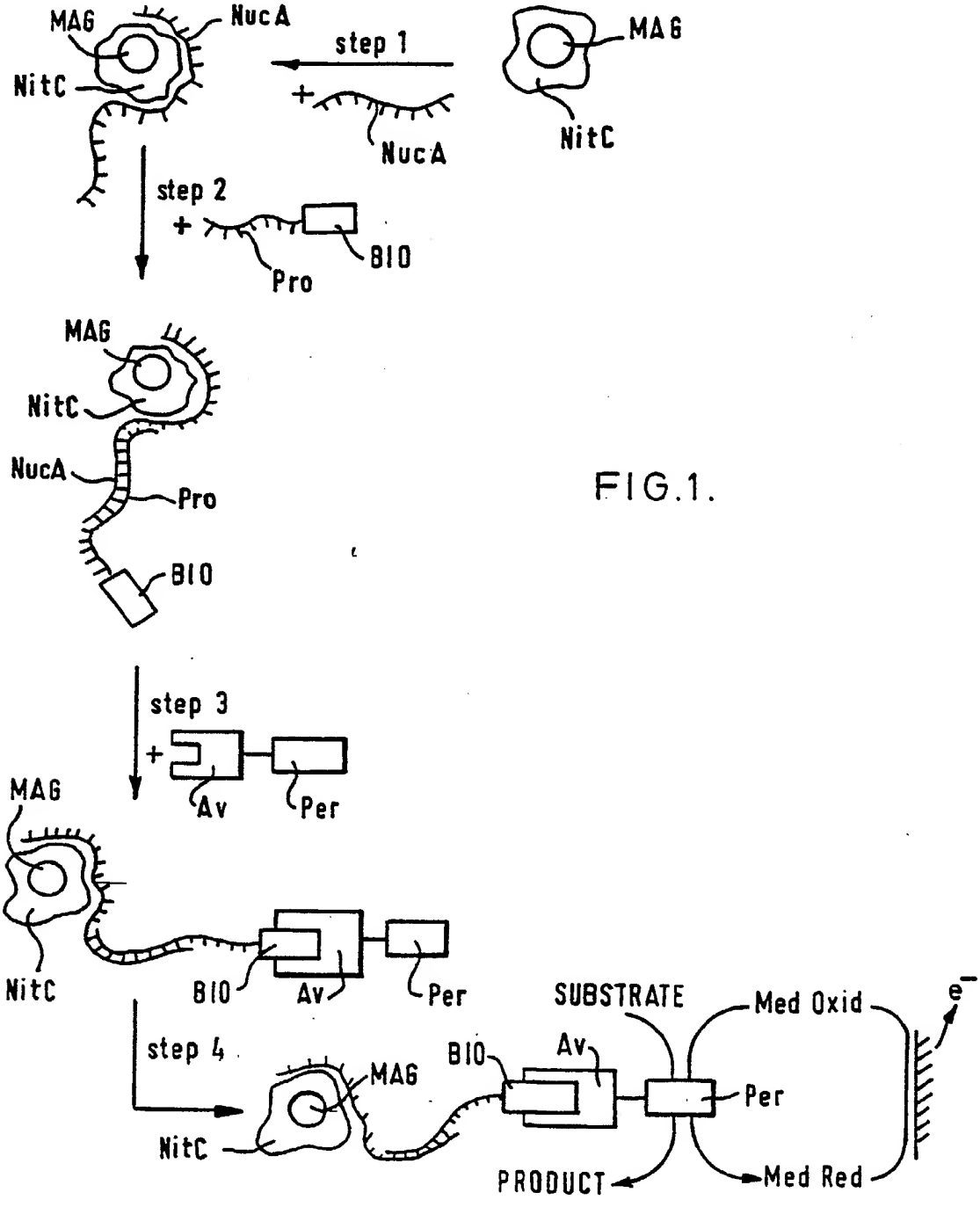


FIG.1.

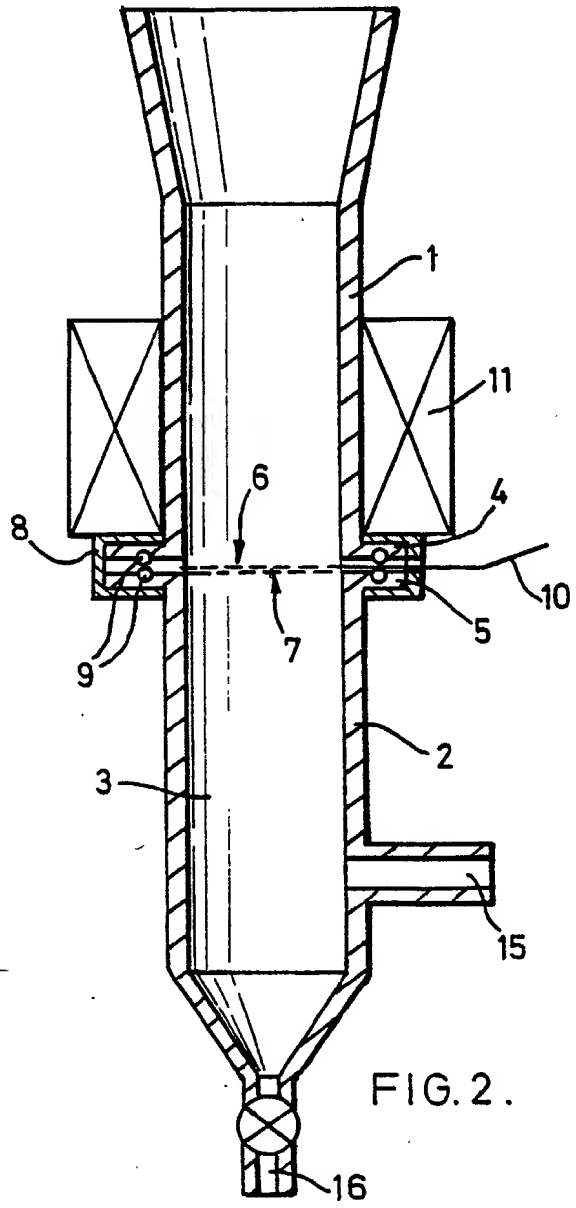


FIG. 2.

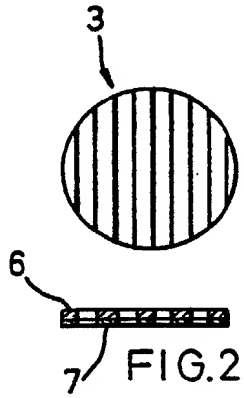


FIG.2a.

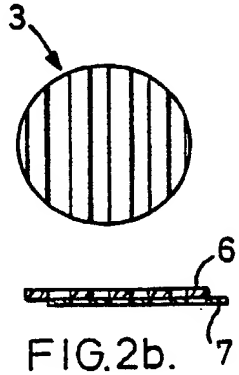


FIG.2b.

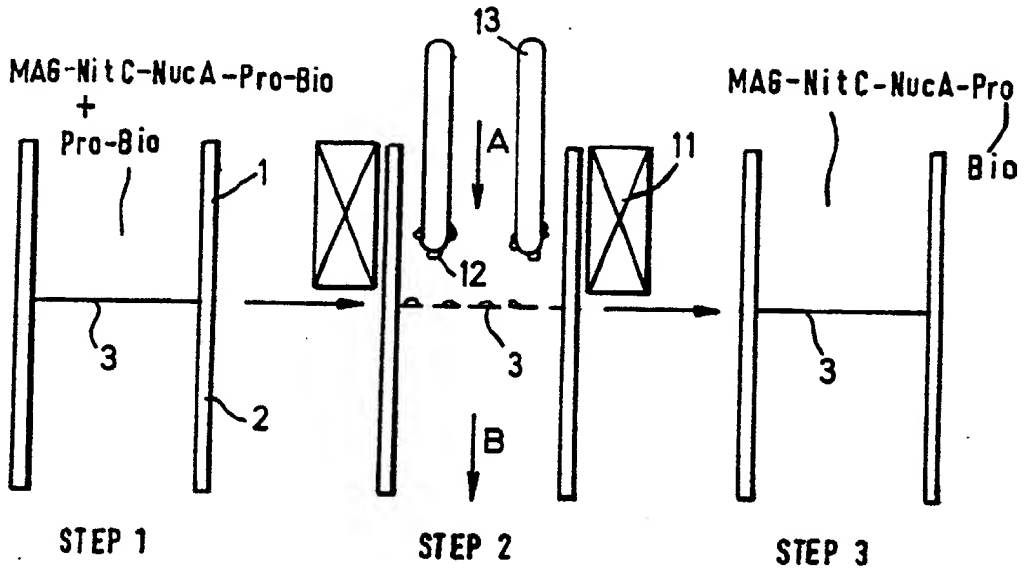

MAG-NitC-NucA-Pro-Bio
+
Pro-Bio
1
2
3
13
A
12
B
11
MAG-NitC-NucA-Pro
Bio
STEP 1
STEP 2
STEP 3

FIG.3.

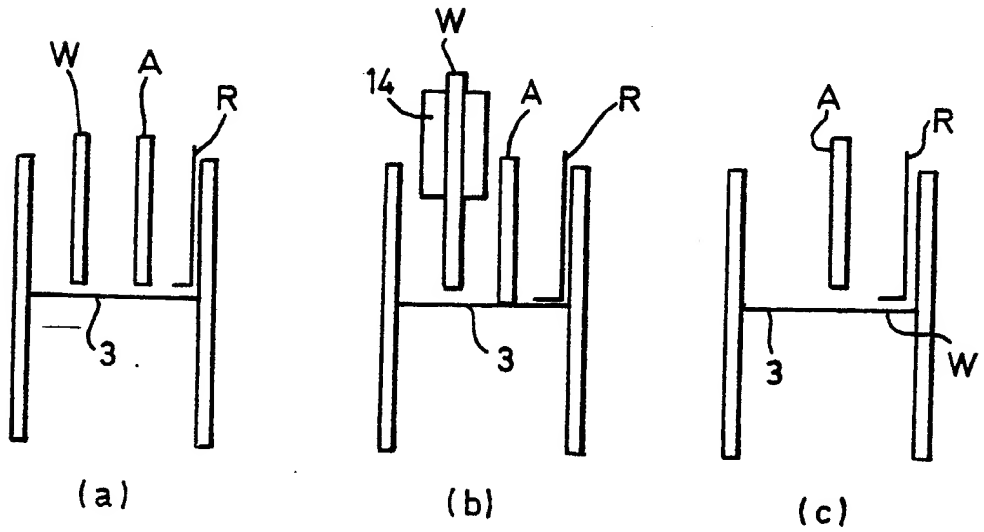

W
A
R
3
(a)
W
14
A
R
3
(b)
A
R
3
W
(c)

FIG.4.

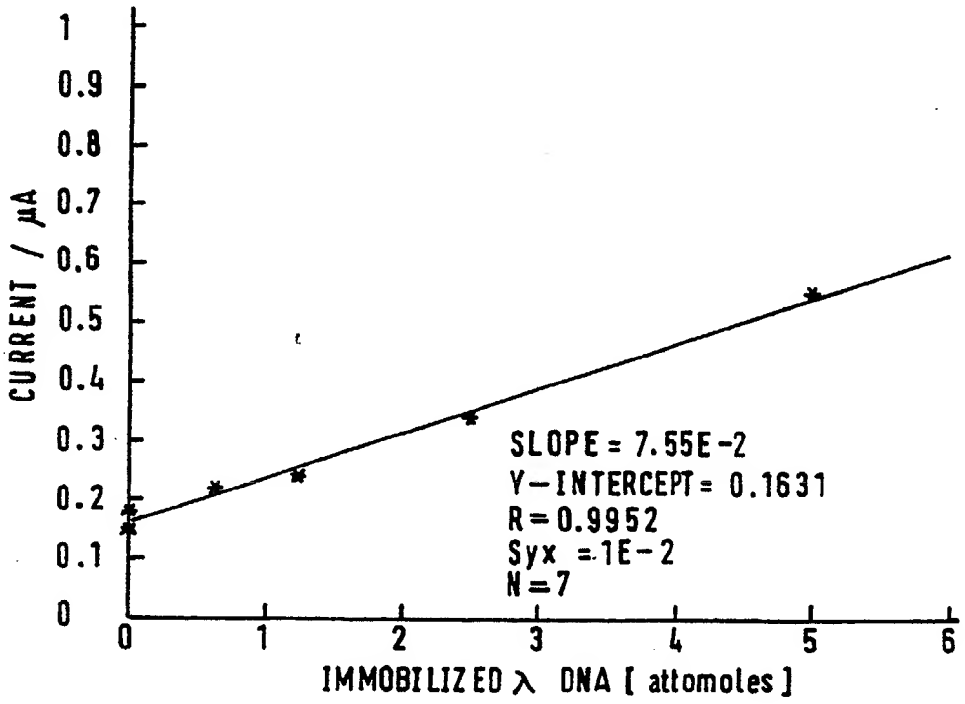

CURRENT / μA
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
IMMOBILIZED λ DNA [attomoles]
SLOPE = 7.55E-2
Y-INTERCEPT = 0.1631
R = 0.9952
Syx = 1E-2
N = 7

FIG. 5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00174

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 Q 1/68; 1/00; G 01 N 33/50; 33/48; C 07 H 19/067

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 Q; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	Chemical Abstracts, vol. 101, no. 17, 22 October 1984 (Columbus, Ohio, US) J. Langdale et al.: "Magnetic DNA", see page 349, abstract no. 147252w, & Biochem. Soc. Trans. 1984, 12(4), 693-4, see the whole abstract	1-24
P,Y	GB, A, 2152664 (SERONO DIAGNOSTICS LTD.) 7 August 1985 see abstract; pages 2-3; claims 1-23	1-24
A	EP, A, 0030087, (TECHNICON INSTRUMENTS COMPANY) 10 June 1981	
Y	EP, A, 0125995 (ADVANCED MAGNETICS, INC.) 21 November 1984 see abstract; pages 32,33	1-24
P,Y	EP, A, 0149339 (GENETICS INTERNATIONAL INC.) 24 July 1985 see abstract; claims 1-6,14-42	17-22

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* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th June 1986	-7 OCT 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EP, A, 0125139 (GENETICS INTERNATIONAL INC) 14 November 1984 see abstract; examples 3a,3b,3c; claims 1,15	17-22
Y	EP, A, 0070687 (STANDARD OIL COMPANY) 26 January 1983 see abstract; page 4, lines 4-28	3,6-8

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-24 : Magnetic nucleic acid particles; use in the assay for nucleic acid.
Claims 25-27: Apparatus for use with magnetic particles.
Claims 28-30: Ferrocene labelled nucleotides per se.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
1-24

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00174 (SA 12721)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/09/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
GB-A- 2152664	07/08/85	None		
EP-A- 0030087	10/06/81	None		
EP-A- 0125995	21/11/84	JP-A-	60001564	07/01/85
		US-A-	4554088	19/11/85
EP-A- 0149339	24/07/85	EP-A-	0125139	14/11/84
		AU-A-	2775384	08/11/84
		WO-A-	8502627	20/06/85
		AU-A-	3832985	26/06/85
		JP-T-	61500706	17/04/86
EP-A- 0125139	14/11/84	AU-A-	2775384	08/11/84
		EP-A-	0125136	14/11/84
		EP-A-	0125137	14/11/84
		EP-A-	0125867	21/11/84
		AU-A-	2775484	08/11/84
		JP-A-	60017345	29/01/85
		JP-A-	60017346	29/01/85
		JP-A-	60017347	29/01/85
		JP-A-	60017360	29/01/85
		AU-A-	2775184	31/01/85
		AU-A-	2775284	31/01/85
		WO-A-	8502627	20/06/85
		EP-A-	0149339	24/07/85
		AU-A-	3832985	26/06/85
		JP-T-	61500706	17/04/86
		EP-A-	0127958	12/12/84
EP-A- 0070687	26/01/83	JP-A-	58040099	08/03/83
		CA-A-	1180647	08/01/85

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82